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Research Note

Detection of *Cryptosporidium parvum* Oocysts on Fresh Vegetables and Herbs Using Antibodies Specific for a *Cryptosporidium parvum* Viral Antigen

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ABSTRACT

The purpose of this study was to determine if the viral symbiont of *Cryptosporidium parvum* (CPV) sporozoites could be used as a target for sensitive detection of the parasite in food samples. Polyclonal sera specific to the recombinant viral capsid protein (rCPV40) was used in a dot blot hybridization assay to detect oocysts recovered from green onions and cilantro. Small batches of chopped green onions and cilantro leaves were artificially contaminated with three different concentrations of oocysts: 10^6, 10^2, and 10^1. rCPV40 was superior in detecting oocysts compared with other antibodies directed toward total oocyst protein and oocyst surface antigens. This study provides evidence that CPV is an excellent target for sensitive detection of *C. parvum* oocysts in foods.

Cryptosporidium parvum is an apicomplexan protozoan parasite that is well documented as a cause of large waterborne outbreaks *(12, 14, 25, 30, 38)*. The transmissive stage of *C. parvum* is the oocyst, which when carried in the feces of humans and companion or domestic animals and wildlife can contaminate surface water *(22, 23, 33, 34)*. Ingestion of the infectious thick-walled oocysts can result in a gastrointestinal illness that lasts 1 to 2 weeks in previously healthy individuals or indefinitely in immunocompromised individuals. *Cryptosporidium* oocysts remain viable after passing through the intestines of animals and unlike other parasitic oocysts are immediately infectious on release into the environment *(28)*. One survey reported that between 24 and 100% of surface waters in the United States contain *C. parvum* oocysts *(40)*. Prevention and detection of *C. parvum* oocysts are essential, because there are no effective drugs or disinfectants available to destroy the parasite. Because this protozoan parasite is a serious issue for the water industry *(1, 23)*, it is also an issue for the fresh produce industry, since contamination via contaminated irrigation waters may occur. The potential for contamination by irrigation waters is enhanced by the fact that irrigation waters can easily vary in their contamination levels and infection requires relatively few oocysts *(36)*. Previous studies identified *C. parvum* oocysts on more than 14% of randomly sampled vegetables in Norway, Peru, and Costa Rica *(27, 29, 31)*. Contamination has been shown to occur to varying degrees with drip or spray irrigation using naturally contaminated source water *(1)*. In fact, this previous study revealed that oocysts could be isolated not only on crops above ground but also within 60 cm of soil depth following drip irrigation using contaminated water. Furthermore, it has been suggested that the outbreaks of the related protozoan parasite *Cyclospora* associated with raspberries that began in 1996 might have originated as contaminated surface water was sprayed onto the surface of the fruit-bearing plants *(2, 16, 37)*.

In general, reports of foodborne outbreaks associated with fresh produce have increased during the past 10 years. This is due to a combination of factors, including increased consumption overall, increased imports of fresh produce from countries that may not have adequate biosafety control measures, and improved molecular detection of pathogens in conjunction with better reporting of foodborne illness. During the past few years, outbreaks of cryptosporidiosis have been associated with foods, including inadequately pasteurized milk and raw milk *(9, 13)*, apple cider *(26)*, green onions *(7)*, cold chicken salad, sausage, and tripe *(5, 6, 21, 36)*. Although causes were determined for these outbreaks, it is possible that other illnesses attributable to *Cryptosporidium* went undetected, because testing is still not routinely performed at many facilities. Available data from documented outbreaks do not exclude the possibility that multiple items at the events may have been contaminated or that the source of contamination is a food worker or cross-contamination during preparation *(7)*.

This study proposes the use of a novel antibody specific for the capsid protein of the double-stranded RNA virus of *Cryptosporidium* (viral symbiont of *C. parvum* [CPV]) for detection. CPV was first identified in 1997 *(19)*,

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but for the most part the function of these viruses remains unclear. The double-stranded RNA virus of interest in the present study was found in all *C. parvum* isolates but not in other *Cryptosporidium* species *(17)*. CPV double-stranded RNA was isolated from *Cryptosporidium hominis* (previously *C. parvum* genotype I) and *C. parvum* genotype II *(18)*.

In a previous study, we successfully used the molecular and antigenic features of the viral symbiont as a target in a novel method for detecting *C. parvum* oocysts in water *(20)*. The relative abundance and stable presence of CPV make it a novel candidate for use in detection applications, because there are approximately 500 viral particles per sporozoite. In theory, this would increase the sensitivity of detecting *C. parvum* oocysts nearly 2,000-fold (four sporozoites per oocyst). Here the antibodies to the recombinant viral capsid protein (rCPV40) were used to detect oocysts recovered from green onions and cilantro, produce items that are most often consumed raw, that are not consistently washed before used, and that have been associated with outbreaks due to contamination by protozoan oocysts *(4, 7, 9, 24)*.

MATERIALS AND METHODS

Parasites. *C. parvum* (Beltsville-1 strain) oocysts were obtained by infecting a 1-day-old calf with 10^6 oocysts. The calf was obtained at birth from the dairy herd at the Beltsville Agricultural Research Center. Feces were collected from days 3 through 10 after infection, pooled, and passed through a series of sieves with increasingly finer mesh, ending with a 45-μm sieve. Sieved fecal material was mixed equally with 2 M sucrose and subjected to centrifugation (60 min, 9,000 × *g*). The supernatant that contained oocysts was diluted in water and then subjected to continuous flow centrifugation. The pellet was suspended in water, then layered onto a discontinuous cesium chloride gradient of 1.05 g/cm^3, 1.1 g/cm^3, and 1.4 g/cm^3, and then centrifuged for 60 min at 9,000 × *g*. The oocysts were aspirated from the interface and suspended in distilled water, stored at 4°C, and used within 6 months after collection.

Production of rCPV40 and immunization of rabbits for production of antisera against recombinant protein. Expression of rCPV40 was performed using the pET28 vector (Novagen, Madison, Wis.). The recombinant plasmid (generously provided by S. Upton) was introduced into *Escherichia coli* BL21(DE3) cells (Novagen) by standard transformation procedures *(15)*. A time course study to identify the time of isopropyl-β-D-thiogalactopyranoside (IPTG) induction, leading to peak expression of recombinant protein, indicated that a 4-h induction was optimal. rCPV40 was isolated by lysing IPTG-induced *E. coli* with denaturing column binding buffer (0.2 M $NaPO_4$, pH 7.8, 0.5 M NaCl, 8 M urea; Invitrogen, Carlsbad, Calif.) and purified by passage over nickel-affinity resin followed by elution with denaturing column elution buffer (0.2 M $NaPO_4$, pH 4.0, 0.5 M NaCl, 8 M urea; Invitrogen). Protein samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to confirm the purity of eluted recombinant protein. Two New Zealand White rabbits (Covance, Denver, Pa.) were each immunized twice during a 6-week period by intramuscular injection with recombinant protein in phosphate-buffered saline (PBS) that contained ImmunoMax SR adjuvant (Zonagen, The Woodlands, Tex.). The rabbits were bled for serum by central articular artery puncture 2 weeks after the last booster immunization.

Inoculation of produce samples. Intact cilantro leaves and green onion samples were washed thoroughly and cut into 5-g and 10-g samples of intact leaves and 2-cm cut pieces, respectively. These weights were chosen for their similar volume size within a 50-ml conical tube. Samples were allowed to dry at room temperature in a biological safety cabinet for 15 min. Samples were then spot inoculated with 10^6, 10^2, or 10^1 of *C. parvum* oocysts in a total volume of 100 μl of sterile water and then air dried again in a safety cabinet for 15 min. Produce samples were washed with the addition of 10 ml of sterile PBS to the 50-ml conical tubes that was then placed on an elliptical shaker for 45 min at 100 rpm. Wash liquid was then recovered by centrifugation at 4,000 × *g* for 10 min. All experiments were performed in triplicate, and three batches of cilantro and green onions were used.

Immunoblotting of recombinant viral capsid protein and vegetable-herb washes. Wash solution was removed and the pellet was resuspended in 1 ml of PBS. From this, 100-μl samples were applied to Immobilon membrane (Millipore, Inc., Bedford, Mass.) using a dot blot apparatus (Bio-Rad, Hercules, Calif.). The impregnated membranes were rinsed briefly with PBS and then immersed in PBS that contained 2% nonfat dry milk to block nonspecific-antibody binding in subsequent steps. After blocking, the membranes were incubated for 1 h with a 1:2,500 dilution of rabbit antiserum to the rCPV40, native *C. parvum* oocysts, or other recombinant *C. parvum* proteins (a 41-kDa oocyst surface protein and total oocyst protein) in PBS that contained 0.05% Tween 20. The membranes were then probed for 1 h with a biotinylated goat anti-rabbit immunoglobulin G (heavy and light chain specific, Vector Laboratories, Burlingame, Calif.), followed by a 1-h incubation with avidin-alkaline phosphatase (Sigma, St. Louis, Mo.), and a final incubation in phosphatase substrate (0.38 mM 5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt, 2.45 mM nitroblue tetrazolium chloride in alkaline phosphatase buffer, 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5) to visualize antibody binding. Membranes were washed with PBS that contained 0.05% Tween 20 three times for 5 min each between each incubation step, and an additional final wash with PBS before substrate was applied.

RESULTS AND DISCUSSION

Cilantro and green onions were inoculated with three concentrations of oocysts, 10^6, 10^2, and 10^1. By immunoblotting using a dot blot system (Fig. 1), the anti-rCPV serum indicated positive detection of all concentrations of oocysts in water applied directly to the membrane. The anti-rCPV serum also detected oocysts recovered from the cilantro and green onion washes for all three levels of inoculum. Recovery of inoculum was optimized following the protocol developed by Endley et al. *(11)* for sample size, inoculum agitation, and recovery. Recovery was not the main issue in this study but rather the ability of the detection assay to work with food substances; therefore, the recovery efficiency was not directly determined. That being said, several factors to optimize recovery efficiency were considered to examine the assay more closely, including the use of small sample weight and volume, along with the use of an isolated container *(32)*. It is postulated that oocyst recovery varies, depending on uncontrollable factors, such

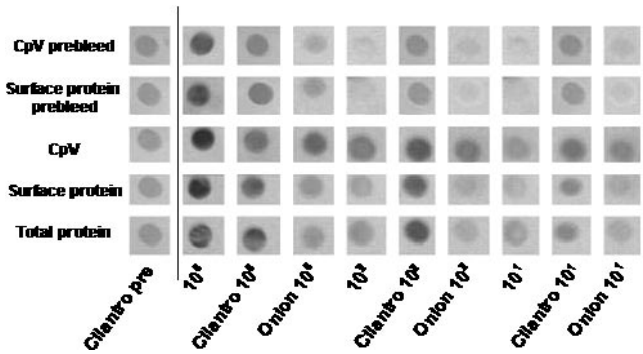

FIGURE 1. *Comparison of immuno-dot blotting signal of* C. parvum *oocyst recovery from cilantro and green onions. Dot blots as labeled horizontally along the bottom were probed with antibodies shown vertically on the left side. Antibodies include anti-rCPV40 preimmunization serum (CPV prebleed), anti–*C. parvum *oocyst surface protein preimmunization serum (surface protein prebleed), anti-CPV40 postimmunization serum (CPV), anti–*C. parvum *oocyst 41-kDa surface protein postimmunization serum (surface protein), or anti–*C. parvum *oocyst total protein postimmunization serum (total protein). Results shown are representative of what was observed throughout five experiments.*

as turbidity and the physicochemical properties of the sample *(5)*. All were relevant issues with both samples tested in this study. Water used to wash cilantro leaves after inoculation was green in color due to pigment released from the cilantro leaves. This affected the subsequent colorimetric determination of the assay and reinforced the need to run control uninoculated samples with each experiment (Fig. 1, far left lane). The material from the washed green onions had the consistency of mucopolysaccharides, similar to that observed and described with bean sprouts by Robertson and Gjerde *(32)*. Both of these traits were worsened by mechanical stomaching or rubbing but were still present as samples were manipulated and washed in the conical tubes before recovery.

For oocysts recovered from the cilantro and green onions, the sensitivity of anti-rCPV serum was greater than that observed with antiserum to total oocyst protein extract or to a 41-kDa oocyst surface protein. The later two antibodies are fairly equivalent in their detection of oocysts directly applied to the membrane. Some nonspecific binding and high background levels were observed when 10^6 oocysts were applied directly to the membrane and similarly for the supernatant recovered from cilantro inoculated with 10^6 oocysts. During the initial analysis of this procedure, uncontaminated pure water samples and samples inoculated with the waterborne protozoa *Trichomonas* were both negative for CPV detection *(20)*. This reinforces the relative sensitivity of this assay. Some reactivity observed with the prebleed sera may be due to the fact that the recombinant protein was purified from *E. coli* and prior exposure to *E. coli* by the rabbits. Because *C. parvum* oocysts are most likely to contaminate foods at lower levels and some oocysts may be lost during removal procedures, it is generally thought that better optimized methods are needed for sensitive detection *(8)*. The immunoblot detection analysis shown in this study may provide such a method.

The use of CPV for oocyst detection is similar to using an indicator organism; however, it is more specific and directly correlates with the pathogen. This is not the case with traditional indicator organisms, such as coliforms. The use of CPV has several advantages. It is always present in *C. parvum* oocysts and appears to be specific for oocysts that are infective to humans (*C. parvum* and *C. hominis*) *(39)*. Perhaps the greatest advantage of the CPV is the ease of detection of CPV due to its abundance in each oocyst. CPV is easily detected using a dot blot system as the oocysts break open or are damaged after being vacuumed onto the membrane; this process exposes the sporozoites and viral particles inside the oocyst. The CPV capsid protein appears to be a good target for detecting *C. parvum* oocysts in the food samples tested in this study. As few as 10 oocysts were detectable after recovery from both green onions and cilantro using antiserum against rCPV40 antibody. This is an important consideration, since the infectious dose ranges from only 1 oocyst up to 30 oocysts *(10)*.

Detection of protozoan parasites is especially difficult, because they do not grow within the food product and enrichment before detection is not possible. Produce items like vegetables and herbs can be contaminated either accidentally or deliberately (bioterrorism). Currently, of the techniques that have been described for detecting *Cryptosporidium* oocysts, most (e.g., immunofluorescence staining) are neither species specific nor sensitive enough to detect extremely low contamination levels. It is possible that filtration, sucrose centrifugation, or immunomagnetic separation systems can be used to concentrate oocysts from liquid samples before microscopy *(3, 32)* or other detection techniques, including immunoblotting. These techniques are timely and can be costly. Although PCR is a potential alternative, some food products contain PCR-inhibiting compounds *(35)*. These techniques may be complex (e.g., microscopy), whereas others are impractical and cost prohibitive (e.g., cell culture) for use in a food quality laboratory. This identification method may well allow for the detection of *C. parvum* from a wide range of samples. The CPV assay should provide food quality and public health laboratories an excellent alternative to immunofluorescence and PCR assays for detecting *C. parvum* oocysts. Use of an immunoblot method for detecting CPV represents a significant advance in this effort, because it is amenable to a dipstick-type assay and does not rely on expensive equipment for analyzing samples.

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